

MOLYBDENUM NUTRITION OF ALFALFA¹

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(WITH TWO FIGURES)

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Molybdenum is the latest element to be found essential for microorganisms and higher plants. The literature, recently reviewed by MULDER (12), indicates that Mo is necessary for N fixation by *Azotobacter* and *Rhizobium* and for the metabolism of nitrates by all plants. The biological importance of Mo was originally discovered by BORTELS (3, 4), who reported that *Azotobacter* and peas, soybeans, and red clover showed increases in growth and in N fixation from additions of Mo. Essentiality of Mo for higher plants was first demonstrated by Arnon and Stout in 1939. Using highly purified nutrient solutions, they produced Mo-deficiency symptoms in tomatoes in six successive experiments. Later JENSEN and BETTY (9) and JENSEN (10) reported that small applications of Mo to alfalfa and clover growing in pots of sand increased the yields of tops and the N content of both tops and roots. Mulder showed that nodulated peas grown in culture solutions lacking both Mo and combined N quickly developed N-deficiency symptoms. The Mo requirement of tomatoes was greatly reduced when ammonium N was substituted for nitrate N in the nutrient solutions. Recent work by HEWITT and JONES (8) indicates that Mo is indispensable for the growth of tomatoes, cauliflower, cabbage, and mustard and that it functions in the reduction of nitrates. VANSELOW and DATTA (15), using the spectrographic method, studied the Mo requirements of lemons and found that leaves containing 0.01 p.p.m. Mo were deficient but those containing concentrations greater than 0.024 p.p.m. were normal. Deficiency was produced with either nitrate or ammonia N.

Further investigation of the importance of Mo in plant nutrition has been stimulated by reports of Mo-deficient soils in AUSTRALIA (1, 2), CALIFORNIA (15) and, more recently, in NEW JERSEY (5, 7). In studying the Mo status of New Jersey crops and soils, an effort was made to obtain additional information on Mo-deficiency symptoms, the quantities of Mo required, and the effects of Mo additions on the Mo and N content of the plants. Special purification and cultural techniques were developed for studying the Mo nutrition of alfalfa.

Purification and analytical methods

Although Mo is not ordinarily added to nutrient solutions prepared from distilled water and analytical grade chemicals, normal plant growth usually results. The plants obtain sufficient Mo from the impurities in

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these solutions or from the containers. Analyses of the greenhouse supply of distilled water showed a Mo content of 0.08 to 0.1 part per billion. Samples of C.P. Fe salts contained up to 0.1 p.p.m. Mo. These impurities had to be removed before the Mo requirements of plants could be studied.

The necessary water for the preparation of nutrient solutions was obtained by redistilling the regular supply of distilled water in an automatic pyrex glass still designed in this laboratory. Pyrex glassware was used throughout the experiment. It was cleaned with hot concentrated HCl and then rinsed with redistilled water. All solutions were stored in pyrex-glass bottles that were covered with Petri dishes or beakers.

The basic nutrient solution consisted of 0.005 M $\text{Ca}(\text{NO}_3)_2$ or CaCl_2 , 0.0005 M KH_2PO_4 , 0.002 M MgSO_4 , 0.0025 M K_2SO_4 , 0.25 p.p.m. B as $\text{Na}_2\text{B}_4\text{O}_7$, 0.25 p.p.m. Mn as MnSO_4 , 0.25 p.p.m. Zn as ZnSO_4 , 0.5 p.p.m. Fe as a mixture of FeCl_2 and FeCl_3 , 0.1 p.p.m. Cu as CuSO_4 , and 0.015 p.p.m. Mo as Na_2MoO_4 . The solution had a pH value of 5.1. With the exception of CaCl_2 , the nutrient salts were purified by recrystallizing C.P. salts twice from redistilled water. The recrystallization was accomplished by saturating boiling water with the salt, and then filtering and cooling. Yields were enhanced in some cases by adding glass-distilled ethanol. Since CaCl_2 is difficult to recrystallize, it was prepared by neutralizing freshly precipitated CaCO_3 , made from Na_2CO_3 and CaCl_2 solutions, with glass-distilled HCl. Stock solutions, 0.5 M in concentration, were prepared from all the macro-nutrient salts, each of which was further purified by the 8-hydroxyquinoline coprecipitation process described by HEWITT and JONES (8). Soluble quinolates left in the filtrate were extracted with redistilled chloroform. With the exception of Fe, stock solutions of all micronutrients were prepared and used without additional purification.

Since Fe salts were known to contain Mo, they were further purified by the following method:

A solution containing 1500 p.p.m. Fe was prepared from recrystallized FeSO_4 and placed in a two-liter separatory funnel. The acid concentration was adjusted to 5% with redistilled HCl, then 0.6 gm. KSCN was added for each 100 ml. of solution. Saturated Na_2S solution was added as a reducing agent until the color due to $\text{Fe}(\text{SCN})_3$ disappeared. The Mo-thiocyanate complex was removed by three extractions with redistilled isopropyl ether. The aqueous solution of Fe was warmed and filtered and $\text{Fe}(\text{OH})_2$ was precipitated with redistilled NH_4OH . The $\text{Fe}(\text{OH})_2$ was washed free of KSCN and neutralized with redistilled 1-N HCl until the pH value was between 2 and 3. The concentration of Fe in the resulting mixture of FeCl_2 and FeCl_3 was determined by analysis, and a stock solution containing 1000 p.p.m. Fe was prepared by dilution. Titration with KMnO_4 showed that 76% of the total Fe was in the divalent form.

After the alfalfa had been grown to the early bloom stage, the tissues were harvested and oven-dried at 70° C, ground in a Wiley mill (check analyses showed no contaminating effect), and analyzed for Mo by a modi-

fication (6) of the thiocyanate stannous chloride method. Total N was determined by the Kjeldahl method modified to include nitrates. Nitrate N was determined by the method described by SNELL and SNELL (12).

Cultural methods

Four-liter pyrex glass beakers, fitted with one-fourth-inch drain holes, were used as culture vessels. These were filled with acid- and alkali-washed pyrex glass cullet crushed to pass a one-fourth-inch screen. Twenty-five gm. of acid-washed pyrex glass wool was mixed with the cullet in each beaker to increase the water-holding capacity. The crushed glass was leached with 1-N redistilled HCl and with redistilled water until the pH value of the effluent was above five. The culture vessels were placed inside two-gallon ceramic jars fitted with "Vinylite" covers (fig. 1). The

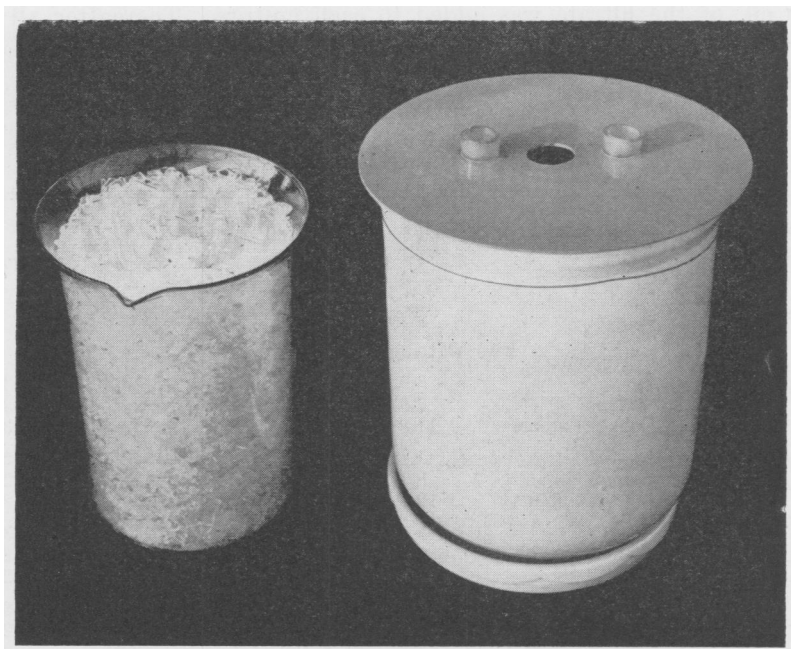


FIG. 1. Culture vessels for study of Mo requirements of alfalfa. The beaker, filled with crushed pyrex glass, was placed inside the ceramic jar.

neck of a 50-ml. Erlenmeyer flask was placed in the center opening of each cover. Each flask had four small holes in its bottom through which the nutrient solutions were dispersed. The flasks were kept covered at all times, except when solutions were being added.

Seedlings of Atlantic alfalfa were grown in pyrex dishes of acid-washed pyrex glass sand and supplied with one-half strength purified nutrient solution without Mo. On February 10, 1949, uniform seedlings approximately four inches in height were transferred to twelve culture vessels. Two plants were supported in each vessel by glass wool fitted in the openings of

the plastic covers. Cultures were divided into four series of triplicate pots and distributed at random on a bench in the greenhouse. Each culture was supplied with one liter a day of purified nutrient solution.

In the first three cuttings, one series was supplied with nutrient solution containing nitrate N and 0.015 p.p.m. Mo. The other three series received identical solutions with the exception of Mo. In the fourth cutting all plants received nitrate N and Mo at rates outlined in table I. After the fourth harvest, all cultures were flushed five times with redistilled water to remove nitrates and the plants were inoculated with *Rhizobium* by the following method: Two nodule masses from an alfalfa plant in the field were washed with redistilled water and macerated in a mortar. Six hundred ml. of purified nutrient solution, without nitrates, was added to the macerated nodules and the solution was filtered through coarse filter paper. Fifty ml. of the filtrate was added to each culture. Thereafter all cultures were supplied with nutrient solutions containing CaCl_2 instead of

TABLE I

EFFECT OF MO ON YIELD,* AND ON MO AND N CONTENT OF ALFALFA SUPPLIED WITH NITRATE N

Mo TREATMENT	PLANT PORTION**	DRY WT.†	Mo††	N CONTENT	
				NITRATE	TOTAL
		gm.	p.p.m.	mg./gm.	%
First Cutting—April 27					
0.015 p.p.m.	Leaves	3.9	1.5	2.62	3.68
	Stems	7.9	1.2	2.31	2.87
	Leaves + stems	11.8	1.3	2.42	3.22
None	Leaves	4.4	0.0	4.65	2.89
	Stems	8.1	0.0	2.79	2.32
	Leaves + stems	12.5	0.0	3.54	2.52
None	Leaves	3.8	0.0	3.32	3.22
	Stems	7.1	0.0	2.61	2.29
	Leaves + stems	10.9	0.0	2.86	2.62
None	Leaves	4.2	0.0	3.38	3.16
	Stems	7.1	0.0	3.17	2.69
	Leaves + stems	11.3	0.0	3.24	2.86
Second Cutting—June 3					
0.015 p.p.m.	Leaves	8.9	5.3	2.22	4.05
	Stems	17.6	3.3	1.85	2.47
	Leaves + stems	26.5	4.0	1.97	3.01
None	Leaves	6.4	0.0	2.91	3.42
	Stems	12.9	0.0	2.43	2.01
	Leaves + stems	19.3	0.0	2.58	2.48
None	Leaves	5.6	0.0	2.98	3.47
	Stems	11.3	0.0	2.66	1.81
	Leaves + stems	16.9	0.0	2.76	2.36
None	Leaves	5.3	0.0	3.01	3.36
	Stems	13.1	0.0	2.88	1.62
	Leaves + stems	18.4	0.0	2.92	2.12

TABLE I (Con't)

Mo TREATMENT	PLANT PORTION**	DRY WT.†	Mo††	N CONTENT	
				NITRATE	TOTAL
		gm.	p.p.m.	mg./gm.	%
Third Cutting—July 5					
0.015 p.p.m.	Leaves	9.6	8.9	2.40	3.94
	Stems	10.6	4.9	2.05	1.68
	Leaves + stems	20.2	6.8	2.22	2.75
None	Leaves	6.6	0.0	2.81	3.30
	Stems	6.7	0.0	2.66	1.40
	Leaves + stems	13.3	0.0	2.73	2.35
None	Leaves	6.7	0.0	2.82	3.44
	Stems	7.3	0.0	2.41	1.71
	Leaves + stems	14.0	0.0	2.61	2.54
None	Leaves	6.1	0.0	2.94	3.36
	Stems	6.9	0.0	2.72	1.76
	Leaves + stems	13.0	0.0	2.82	2.51
Fourth Cutting—July 27					
0.015 p.p.m.	Leaves	3.7	7.4	2.49	4.50
	Stems	4.1	4.2	2.00	2.04
	Leaves + stems	7.8	5.7	2.23	3.22
0.10 p.p.m.	Leaves	2.8	9.8	2.52	4.04
	Stems	3.6	5.6	2.18	1.71
	Leaves + stems	6.4	7.4	2.33	2.73
None	Leaves	2.6	0.0	3.46	3.75
	Stems	3.1	0.0	2.65	1.68
	Leaves + stems	5.7	0.0	3.01	2.62
0.001 p.p.m.	Leaves	3.0	0.2	2.41	4.52
	Stems	3.5	0.1	2.25	1.76
	Leaves + stems	6.5	0.1	2.32	3.03

* First cutting: no significant difference in yield.

Second cutting: increase in yield of leaves + stems from Mo treatment significant at 2% level.

Third cutting: increase in yield of leaves + stems from Mo treatment significant at 1% level.

Fourth cutting: L.S.D. in yield of leaves + stems at 1% level = 1.8 gm., at 5% level = 0.8 gm.

** Petioles are included with leaves.

† Mean yield of three cultures.

†† 0.0 = less than 0.1 p.p.m. Mo.

Ca(NO₃)₂ and Mo at rates given in table II. The fifth and sixth cuttings were grown on atmospheric N.

Responses when supplied with nitrates

No yield responses were obtained from Mo additions in the first cutting (table I) but the untreated plants exhibited deficiency symptoms. They were generally light green and their lower leaves were chlorotic. After the first cutting was harvested, Mo-treated plants grew more rapidly, were darker green, and appeared more vigorous than those receiving no Mo. At the time of the second harvest, plants without Mo had devel-

oped severe chlorosis and a scorched appearance of the lower leaves. Responses to Mo were significant at the 2% level in this cutting. In the third cutting, plants not receiving Mo showed the same deficiency symptoms, and a highly significant increase in yield from Mo was obtained.

After the third harvest, Mo was applied at two additional rates. The fourth cutting was harvested 22 days later, since the intense daytime heat in the greenhouse caused wilting of the leaves. The plants without Mo showed the typical deficiency symptoms, but the treated plants were normal. Additions of Mo at rates of 0.015, 0.10, and 0.001 p.p.m. increased yields 37, 12, and 14%, respectively, over the untreated series. The yield increase for the 0.015 treatment is not comparable with those for the 0.10 and 0.001 Mo treatments, since the former received Mo for the first three cuttings.

The untreated plants contained less than 0.1 p.p.m. Mo in all cases. Identically treated plants harvested early in spring contained less Mo than those that were harvested in June or July. This difference was probably caused by variable light conditions or by an accumulation of Mo in the culture media. The Mo concentration in plants from the fourth cutting showed a close correlation with the quantities of Mo applied. Plants supplied with solutions containing 0.001 p.p.m. Mo contained a mean of 0.1 p.p.m. Mo. Leaves from treated plants consistently contained over 50% more Mo than the stems.

Molybdenum had a pronounced effect on the N content of both leaves and stems. The total N in Mo-treated plants from the first cutting ranged between 13 and 28% higher than that of the untreated plants. This relationship was consistent in all cuttings. In the second cutting, plants receiving Mo contained 13 to 28% more total N than untreated plants, and 8 to 17% more in the third. Additions of Mo at rates of 0.015, 0.1, and 0.001 p.p.m. in the fourth cutting increased the total N concentration of the plants by 23, 4, and 16%, respectively. Plants grown without Mo accumulated nitrates in their tissues, but not to such an extent as has been reported for non-legumes (8).

Responses when grown on atmospheric nitrogen

After the fourth cutting was harvested, the cultures were removed from the jars and the roots were examined for nodules. Many small roots were concentrated on the bottoms and along the sides of the beakers, but no nodules were observed. The beakers were replaced in the jars, flushed with water, and inoculated as previously described. The fifth and sixth cuttings were grown without combined N.

All plants grew very slowly for three weeks after the inoculation and developed typical N-deficiency symptoms. The upper leaves were light green and many of the lower leaves became necrotic and abscised. These symptoms increased in severity until September 23, when plants receiving Mo showed some evidence of recovery. Plants treated with Mo at rates of

0.015, 0.10, and 0.001 p.p.m. rapidly regained their normal color and succulence, but those without Mo remained chlorotic. By August 24, necrosis had developed on the margins of many of the upper leaves of untreated plants. The symptoms differed from those of N-deficiency. The third cutting was harvested on August 30. The series receiving Mo at rates of 0.015, 0.10, and 0.001 p.p.m. yielded 71, 63, and 63% more dry matter, respectively, than the untreated series (table II). These responses were highly significant.

TABLE II

EFFECT OF APPLICATIONS OF Mo ON YIELD* AND ON Mo AND N CONTENT OF ALFALFA GROWN ON ATMOSPHERIC N

Mo TREATMENT	PLANT PORTION**	DRY WT.†	Mo††	TOTAL N
		gm.	p.p.m.	%
Fifth Cutting—August 30				
0.015 p.p.m.	Leaves	3.7	6.7	3.75
	Stems	3.3	3.1	1.65
	Leaves + stems	7.0	5.0	2.77
0.10 p.p.m.	Leaves	3.6	13.2	4.36
	Stems	3.1	6.3	1.57
	Leaves + stems	6.7	10.0	3.07
None	Leaves	2.1	0.0	2.54
	Stems	2.0	0.0	1.20
	Leaves + stems	4.1	0.0	1.88
0.001 p.p.m.	Leaves	3.6	0.6	4.06
	Stems	3.1	0.3	2.24
	Leaves + stems	6.7	0.5	3.21
L. S. D. in yield of leaves + stems at 1% level = 1.2 gm., at 5% level = 0.8 gm.				
Sixth Cutting—October 14				
0.015 p.p.m.	Leaves	9.0	6.2	4.03
	Stems	6.6	3.5	2.26
	Leaves + stems	15.6	5.1	3.28
0.10 p.p.m.	Leaves	9.8	11.3	4.17
	Stems	6.3	6.7	2.10
	Leaves + stems	16.1	9.5	3.36
None	Leaves	2.0	0.0	2.24
	Stems	1.6	0.0	1.15
	Leaves + stems	3.6	0.0	1.76
1 microgram§	Leaves	3.9	0.0	3.42
	Stems	3.8	0.0	1.90
	Leaves + stems	7.7	0.0	2.67
0.001 p.p.m.	Leaves	10.3	0.3	4.06
	Stems	6.0	0.1	2.18
	Leaves + stems	16.3	0.2	3.36

* Fifth cutting: L. S. D. in yield of leaves + stems at 1% level = 1.2 gm., at 5% level = 0.8 gm.

Sixth cutting: L. S. D. in yield of leaves + stems at 1% level = 2.2 gm., at 5% level = 1.2 gm.

** Petioles included with leaf portion.

† Mean yield for three cultures, except the no Mo and 1 microgram treatment in the sixth cutting.

†† 0.0 = less than 0.1 p.p.m. Mo.

§ One microgram Mo added after fifth harvest.

Immediately after the fifth harvest, one deficient culture was treated with one microgram Mo and, thereafter, it was flushed with a purified nutrient solution containing no Mo. For two weeks after treatment, the plants in this culture grew normally, with no evidence of deficiency symptoms. During the same period the plants in untreated cultures developed a general chlorosis of their upper leaves and a pronounced necrosis of many lower leaves (fig. 2). On October 14, the deficient plants showed necrosis

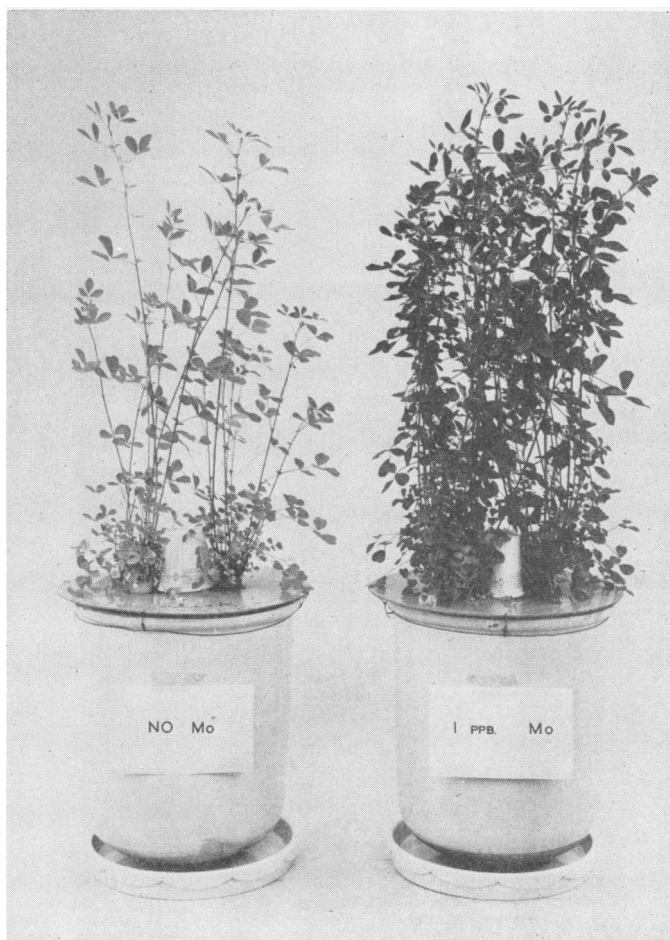


FIG. 2. Effect of Mo on growth of Atlantic alfalfa utilizing atmospheric N. Left, no Mo; right, 1 part Mo per billion of solution.

of the tips and margins of many upper leaves, but no deficiency symptoms were apparent in any of the Mo-treated plants. This was considered conclusive evidence that the symptoms observed in untreated cultures were caused by Mo deficiency and not by lack of inoculation.

In the sixth cutting, plants receiving Mo at rates of 0.015, 0.10, and 0.001 p.p.m. yielded between 334 and 352% more dry matter than the un-

treated plants. The plants that were treated with only one microgram Mo produced 114% more dry weight than those receiving no Mo. Under the conditions of this experiment, 0.001 p.p.m. Mo in nutrient solutions was required for alfalfa grown on atmospheric N and was sufficient for maximum yield.

The analytical data for the fifth and sixth cuttings show the same correlation between quantities of Mo added and the Mo content of the plants as was observed in the first four cuttings. Deficient plants in both cuttings contained less than 0.1 p.p.m. Mo in either stems or leaves. The plants treated with Mo at the rate of 0.001 p.p.m. had a mean Mo content of 0.5 p.p.m. in the fifth cutting and of 0.2 p.p.m. in the sixth. These concentrations in tissues were sufficient for maximum yield and N fixation. No Mo was detected in plants from cultures receiving one microgram Mo, yet they showed marked response to this small addition.

All Mo-treated plants in both the fifth and sixth cuttings contained more total N than the untreated plants. The increases for the 0.015, 0.10, and 0.001 p.p.m. Mo applications were 47, 63, and 71%, respectively, in the fifth cutting, and 86, 91, and 92% in the sixth. Plants treated with one microgram Mo contained 52% more total N than those untreated plants. These data provide conclusive evidence of the importance of Mo for N fixation by alfalfa.

Yields and analyses of roots

The roots were removed from the pots on December 2, washed, and examined. Most of the nodules were found on the upper large roots and very few on the fine roots, which were concentrated in the bottoms of the beakers. The data in tables II and III show no correlation between numbers of nodule masses and either yield or N content of roots, stems, and leaves. Since a large number of nodules were found on Mo-deficient roots, it is concluded that the N-fixing bacteria were parasitic on these roots. Roots of plants that had been supplied with Mo produced greater dry weights

TABLE III
EFFECT OF APPLICATIONS OF Mo ON YIELD, NUMBER OF NODULES, AND Mo AND
N CONTENT OF ALFALFA ROOTS
HARVESTED DECEMBER 2.

Mo TREATMENT	NODULES PER CULTURE	DRY WT.*	Mo**	TOTAL N
		<i>gm.</i>	<i>p.p.m.</i>	<i>%</i>
0.015 p.p.m.	31	43.9	8.5	2.01
0.10 p.p.m.	62	40.0	15.0	1.99
None	34	27.6	0.0	1.32
1 microgram†	29	32.0	0.0	1.54
0.001 p.p.m.	53	42.1	0.7	2.07

* Mean yield for three cultures except in the no Mo and one microgram treatment.

** 0.0 = less than 0.1 p.p.m. Mo.

† Immediately after the fifth harvest, this culture was treated with one microgram Mo. Thereafter it received purified solutions containing no Mo.

and contained more total N than those from untreated plants (table III). Additions of Mo to the nutrient solutions produced the same trends in yield and in Mo and N content of roots as were observed for stems and leaves.

Function of molybdenum

Experiments indicate that Mo functions as a biological catalyst in symbiotic-N fixation and in the reduction of nitrates in non-leguminous plants. This study presents evidence that legumes also require Mo for nitrate reduction. The specific reactions catalyzed by Mo are not known. It is possible that accumulation of nitrates in Mo-deficient plants is an indirect effect caused by a lack of Mo in some reaction preliminary to nitrate reduction. Enzyme experiments were conducted on both Mo-deficient and normal alfalfa to study this point.

Young shoots of plants grown after the sixth harvest were ground in a Waring Blendor, extracted with water, and their succinic dehydrogenase activity was estimated by the Thunberg technique (14). Five ml. of extract, representing 0.1 gm. of green tissue, from normal plants reduced 1 ml. of 0.00027 M methylene blue in two hours. An equal amount of extract from a deficient plant failed to completely reduce that quantity of methylene blue in 12 hours. Preliminary studies indicated no consistent difference in the rate of reduction of nitrates by nitrate reductase in the two extracts. Since the energy required for nitrate reduction in plants must be supplied by dehydrogenation reactions in respiration, it is concluded that Mo indirectly affects nitrate reduction by enzymatically catalyzing certain dehydrogenation reactions. If this conclusion is correct, then the importance of Mo in Fe metabolism, reported by MILLIKAN (11) might be explained. Mo may be a constituent of certain dehydrogenases while Fe in the cytochrome system acts as a hydrogen acceptor for dehydrogenation reactions.

Summary and conclusions

Cultural techniques and a method for the purification of Fe salts were developed for the study of the Mo requirements of alfalfa in nutrient cultures.

Studies were made of the influence of Mo on yield and the Mo and N content of alfalfa grown with purified culture solutions. Four cuttings were supplied with nitrate N and variable amounts of Mo. In the fifth and sixth cuttings, the Mo nutrition of alfalfa was studied when atmospheric N was utilized.

Plants receiving nutrient solutions containing nitrates and no Mo developed deficiency symptoms as indicated by light-green upper leaves and chlorosis and necrosis of lower leaves.

Mo-treated alfalfa supplied with nitrates produced significantly higher yields than the untreated in three of the four cuttings. There was no con-

sistent response to applications of more than 0.001 p.p.m. Mo in nutrient solutions.

Mo-deficient plants contained less total N and they accumulated nitrates when this form of N was supplied.

There was a close relationship between the quantities of Mo applied in nutrient solutions and the Mo content of the tissues. Mo-deficient plants contained less than 0.1 p.p.m. Mo. The leaves consistently contained about 50% more Mo than the stems.

After nitrates were removed from nutrient solutions and the cultures were inoculated, all plants temporarily developed severe N-deficiency symptoms. Within three weeks, the Mo-treated plants regained their normal color and succulence but the untreated plants remained chlorotic. Many upper leaves of deficient plants later showed necrosis of tips and margins while the lower leaves abscised.

Mo-treated plants grown on atmospheric N produced highly significant increases in yield and contained between 47 and 92% more total N than plants receiving no Mo.

There was no correlation between numbers of nodule masses and yield and N fixation.

The succinic dehydrogenase activity of Mo-deficient plants was much less than that of normal plants supplied with Mo.

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